SZENT ISTVÁN UNIVERSITY

THE EFFECTS OF DIFFERENT CHEMICALS THROUGH MULTIGENERATION EXPOSURE ON ZEBRAFISH (DANIO RERIO) DEVELOPMENT

PhD thesis

Róbert Kovács

Gödöllő

2017

The doctoral school

Name:	Doctoral School of Animal Science
Research field:	Animal husbandry
Director:	Dr. Miklós Mézes professor, MHAS, Szent István University, Faculty of Agricultural and Environmental Sciences, Institute for Animal Sciences, Department of Nutrition
Supervisor:	Dr. Béla Urbányi professor, DSc, Szent István University, Faculty of Agricultural and Environmental Sciences, Institute of Aquaculture and Environmental Safety, Department of Aquaculture
Co-Supervisor:	Dr. Zsolt Imre Csenki-Bakos research fellow, PhD, Szent István University, Faculty of Agricultural and Environmental Sciences, Institute of Aquaculture and Environmental Safety, Department of Aquaculture

…….....

Approval of Head of Doctoral School

…….....
Approval of the Supervisor

…….....
Approval of Co-supervisor

1 Introduction and objectives

1.1 Introduction

The consumption of various medicines has become part of our normal daily routine already. These compounds play important role in the protection of human health and in living a life of higher quality. As much as we can foresee, the consumption of medicines will continue to increase in the future partly due to the global increase in the human population partly due to the longer lifetime of people. As the consumption increases, the quantity of active substances reaching the environment will increase continuously as well.

As the active medical substances can be found in many applications and they are present in our environment permanently they can also be called pseudo-persistent pollutants. Their presence in the environment has been well known for a long time and considering their strong biological effect the question has become inevitable: what impact do they have on the living organisms in the waters and soil and, as a consequence, on us human beings? Earlier, the main sources of the medical substances leaking into the environment were the draining waters of the pharmaceutical companies, but by today the new "greener" technologies have obviously and substantially reduced the volume of contamination originating from these sources. On the other hand however, medicines consumed by people and emitted into the environment as well as medicines disposed of via the waste bins without consuming them reach the natural waters and the soil through the communal sewage and landfills.

The environment risk assessment of antineoplastic compounds reaching natural waters as part of the large groups of pharmaceutical agents has a short history of a few decades only. During this time mainly the presence of various active agents was detected in hospital and communal sewage and they were also detected in nanograms in some natural watercourses, which receive sewage waters. Many of these chemicals are identified as strongly cytotoxic and genotoxic compounds or endocrine disruptors therefore they may pose potential danger to living organisms and these effects can occur even at population level. However, these medicines were not identified as dangerous substances in the environmental risk assessments due to their low concentration or short half-life.

The environmental risk assessment models consist mainly of acute and chronic tests of one or two months and they are based on the results of

chemical analytical stability tests. No actual long term tests are made although a full life cycle test carried out on a vertebrate model or a multigenerational examination might draw up a much more detailed picture about the real environmental risk of the antineoplastic medicines.

The most important vertebrate model organisms of the long term, even multigenerational ecotoxicological tests are fish. One of the main advantages of their use as vertebrate organisms is that they spend their entire life in water therefore they are permanently exposed to contaminants appearing there and they are able to absorb these contaminants all over their body surface. Another important advantage of using fish as model organisms is that the species used in various laboratory tests represent well also the effects contaminants may present on other species kept in aquacultural systems. This is especially important as pond farms are established in many cases on water bases serving as recipients for communal and industrial sewage. Active substances appearing in these water bases may have an impact on the efficiency of the production or they can even be detected in the tissues of the processed animal. Although, these xenobiotics are present in the waters always in some kind of mixtures, it is very important to learn the effects of the individual chemicals separately in the individual environmental concentration ranges.

As to the laboratory fish models, zebrafish (*Danio rerio*) is increasingly becoming the subject of not only the acute, but the long term eco-toxicological tests as well. It is also recommended to use as model organism in OECD and EPA toxicity tests elaborated for fish. This species is often used for early life phase examinations of fish as specified in OECD 210 being important part of the environmental risk assessment of the human pharmacology and veterinary formulations. As opposed to the protocols elaborated for the acute and sub-chronic tests, multigenerational OECD toxicity test protocol is available only for medaka (*Oryzias latipes*) presently. This protocol is definitely aimed at examining the reproduction biology effects of the EDC compounds, but modified methods focusing on other end points or sub-lethal impacts like genotoxicity would be required too.

1.2 Objectives

During my examinations I tested the toxicities of four antineoplastic active agents each with different mode of action using zebrafish models. The goal of my work was to examine the effects of 5-fluorouracil (5-FU), cisplatin (CisP), etoposide (ET) and imatinib mesylate (IM) to the embryonic and post-embryonic development of the animals in case of acute, sub-chronic and chronic exposures. During the acute tests, I wanted to define the LC_{50} values of the substances both on adult fish and embryos. I wanted to examine the acute lethal impact of the mixtures of these medicines on zebrafish embryos as well. Further, in the cases of 5-FU and IM I wanted to study the effect of these compounds in the early life phase of zebrafish at sub-chronic exposure. Beside the above, my goal included also the elaboration of a test, which would enable the researcher to study the toxicity of 5-fuorouracil and imatinib through multigenerational tests at ranges near the environmental concentration.

2 Materials and methods

2.1 Animals used and keeping conditions

The tests were performed in the Department of Aquaculture, Szent István University. AB wild type zebrafish line was used for the examinations. Before starting the tests, I kept the fish in a constant flow recirculation system (Tecniplast Zebtec, Tecniplast, Buguggiate, Italy) with 14 hours light and 10 hours dark lighting pattern. The water temperature in the recirculation system was 25±0.5 °C, with pH of 7.5±0.2 and conductivity of 525±50 µS. The fish were fed twice a day using SDS Small Gran (SDS – Special DietsServices Inc.) fish feed and they received *Artemia spp*. (SERA GmbH.) nauplius larvae as living feed.

In order to obtain zebrafish embryos to be used for the tests I had the adult fish spawn in 1 liter reproduction vessels (Tecniplast). The fish were transferred to the reproduction basins in the afternoon of the day prior to the spawning day. Male and female fish were separated in the basins by transparent polycarbonate wall. The spawning took place in the next morning in a synchronized way by removing all separating sheets in the same time. The selection of the embryos was performed under stereo microscope and it took place following the collection of the eggs. The intact and normally developing embryos were then transferred into the test vessels.

The tests were performed in accordance with the permission for performing animal tests (XIV-I-001/2303-4/2012) titled “Toxicology tests on fish” issued based on Act No. XXVIII. of 1998.

2.2 Acute adult fish test

Acute adult fish tests were performed in accordance with OECD 203 test protocol. Before performing the main test I used a range finding test in order to define the dose range. For this 1, 10 and 100 mg/l solutions of each tested substance were prepared. The preliminary tests were performed in 1 liter polycarbonate tanks, 3 fish were put in each basin. The main test was carried out for all substances except for the ones, where none of the fish treated by 100 mg/l concentration solution died during the test period. The 100 mg/l limit was defined based on OECD 203 protocol. All fish involved in the tests were 6-8 months old. Average body weight of the fish: 0.3±0.1 g, average standard body length: 2.5±0.5cm. Main tests were performed in 3 liter polycarbonate basins static test setup was used for all substances with 5 concentrations. Temperature of the treatment solution during the tests: 24±1 ◦C. For each concentration, the test was repeated twice, 7 fish were put in

each basin. The test time was 96 hours for both the preliminary and the main test. The main test was carried out using CisP and IM. Control groups were treated in system water in identical conditions with the above, in two replicants.

2.3 Acute embryo tests

The embryo tests were planned based on the OECD FET (Fish Embryo Toxicity Test) 2006 Draft guideline. Accordingly, the embryos were transferred into the test vessels 2 hours after fertilization at the latest, following selection, before reaching the 8-cells phase. The duration of the tests were increased to 120 hours so, that as much information as possible could be collected about the effect of the substance. Tests for each substance were done in 24 well tissue culture plates, 20 embryos were examined for each concentration, with two repetitions. The treatment was static. Mortality and occurrence of sub-lethal effects were checked in every 24 hours. For 5-FU and ET solvent-added control groups were established as well.

2.4 Acute tests of mixtures on embryos

Mixture impact tests were performed for two compounds. The two compounds were: CisP and IM. When selecting the concentrations to be used in the mixtures, 96 hours mortality data originating from the acute embryo tests of the two substances were considered.

LC_5, LC_{10}, LC_{20}, LC_{50} and LC_{90} calculated values of 96 hours exposure to both compounds were used for the tests. The treatment solution consisted of a mixture created using half of the concentrations assigned to the same mortality values of the substances. (Cisplatin/Imatinib LC_5, LC_{10}, LC_{20}, LC_{50}, LC_{90}).

The tests of the mixtures were carried out in accordance with the OECD 236 FET test. Instead of the setup recommended by the OECD protocol my tests were performed in 3 steps, using 10 embryos for each repetition. The individual repetitions were placed separately on 2 well tissue culture plate, so one plate contained one repetition of 2 concentrations and 4 control embryos. The test was performed twice. After 96 hours of exposure, the LC_{50} values were calculated based on the test results.

2.5 Calculating the predictive toxicity of a mixture

The predictive toxicities of the mixture were also calculated based on the available acute toxicity results. When doing this, the method of concentration addition was used, which presumes a similar effect for the two

substances and so the same or similar mode of action for the components of the mixtures in the organism. On one hand I used the Concentration Addition-CA (Loew addition) method for defining the estimated toxicity of the mixtures.

Predictive toxicity was also assessed using the method of Independent Action – IA (Bliss Independent Action). When using this method the researcher presumes independent mode of action and different target areas for the two substances.

2.6 Sub-chronic tests on young zebrafish

I took OECD 210 test protocol as guidance for the early life phase test. This test was performed for 5-FU and IM with the main aim to learn sub-chronic effects of these two substances on the early development of the fish. During the tests I strived to examine a wide range of concentrations therefore I used concentrations of 10 and 100 ng/l, 1, 10, 100 µg/l, and 1 as well as 10 mg/l in the case of 5-FU. As for IM, the tested concentrations were: 100 ng/l, 1, 10 and 100 µg/l, as well as 1 and 10 mg/l. When testing 5-FU a solvent-added control group was set up as well in a concentration of 0.01% (100 µl/L). For all treated group, the quantity of DMSO was raised to 100 µl/L. The test lasted for 33 days. For all treated group, 2 repetitions were applied using 50 embryos for each repetition. At the start of the tests (like in the case of the FET test) the embryos were put into the treating solution after a selection process before reaching the 8-cells stage. The treatments were performed in a semi-static system in both cases. In the frames of the early life phase tests the treatment of the embryos and larvae was performed in the beginning in Petri dishes with diameters of 10 cm. The embryos were put every day into clean Petri dishes and prepared fresh solutions. In 5-days old larva stage the larvae were transferred into larger Petri dishes (with diameters of 15 cm). The test solution was changed daily in this case as well. After 15 days and on, the larvae were treated in 1l test vessels until the end of the test. The test solution was changed daily and partially (at least 2/3d of it). The fish were transferred with care into clean vessels and fresh test solutions every other day. The mortality was recorded daily during the entire test period. At the end of the test the fish were over-anesthetized using 0.02 % Tricaine (Tricaine methanesulphonate; CAS. 886-86-2, Sigma-Aldrich) solution, their wet body masses were measured and pictures were made about the fish through microscope for measuring their standard body length using ImageJ software. Both the wet and 24 hours (60°) dry body weights were measured using an analytic scale. Due to their very low individual

weight, the fish were measured in groups of 5 both for wet and dry body mass examinations.

2.7 Multigenerational test of 5-fluororuracil on zebrafish

During the test, the fish were treated in a semi-static system by 3 concentrations namely: 10 ng 5-FU/l, 1 µg 5-FU/l and 100 µg 5-FU/l. When selecting the concentrations, the results of the earlier life phase test and the environmental concentrations described by the literature were considered. All treatment concentrations and the solution of the solvent-added control group as well contained 1 µl DMSO per liter. Beside the solvent-added control group a negative control group was also applied for the test. Two replicants were applied for both treatment groups (groups A and B). The fish were kept at 25±1 °C during the test.

When establishing the examination and deciding the test duration the study published by EPA was considered mainly. The examination is built on a two-generation test setup, which starts with the sub-chronic two weeks long preliminary treatment of generation F0 adult, sexually mature animals. Generation F1 was exposed to the effects of the tested material during its entire lifetime, while the exposure was confined to the early life phase only in the case of generation F2.

2.8 Multigenerational test of imatinib

Like in the case of 5-FU, 3 concentrations with values of 10 ng/l, 1 µg/l and 100 µg/l were used for the multigenerational test of imatinib mesylate. Based on the experiences gathered during the 5-fluorouracil tests several changes were applied to the test design with the main aim to examine the changes taking place in the reproduction ability with a greater emphasis in the case of imatinib.

2.9 Statistical evaluation

Minitab 16 (Minitab, Coventry, United Kingdom) program package was used in the FET tests for defining the LC_{50} values. To calculate LC_{50} values, I used probit analysis also recommended by the protocols at 95% confidence interval. When evaluating sub-lethal dose-effect relations non-linear regression was used and GraphPadPrism 5.0 (GraphPad Software, La Jolla, USA) program package was applied for calculating EC_{50}.

OECD protocol was used as well as a basis for evaluation for the tests carried out on adult fish. Like in the FET test, Minitab 16 (Minitab,

Coventry, United Kingdom) program package was used here as well for probit analysis in order to calculate the LC_{50} values at 95% confidence interval.

The statistical analysis of the mixtures of the tested substances were done using R program drc package (R DevelopmentCore Team 2013). Mortality was calculated from the raw data at 96 hours exposure and logistic fitting was carried out. Conclusions were made at a confidence level of 95% ($p \leq 0.05$) in all cases.

During the examination of early life phase SPSS 14 software package was used to define mortality LOEC and NOEC values. The two concentration limits were defined by independent trials at 95% confidence interval. For the examination of the test parameters, GraphPad Prism 5.0 program was used. For 5-FU, when examining the body masses unpaired t trial was used, while the evaluation of the body length results was performed using one-aspect variance analysis and Tukey's post hoc test.

GraphPad Prism 5.0 program, one-aspect variance analysis and Dunnet's post hoc teste were used to evaluate IM test results at 95% reliability.

Values reached during the multigenerational test were processed using Microsoft Excel program. Conclusions were made at a confidence level of 95% ($p \leq 0.05$) in all cases. Statistical analysis was performed using GraphPad Prism 5.0 statistical program package.

Comparison of the mortality results was carried out using GraphPad Prism 5.0 statistical program package and Fisher exact test was used at 95 % reliability level.

In the case of data showing normal distribution one-aspect variance analysis and Tukey's test as a post-hoc test were performed, while in the case of data showing non-normal distribution Kruskal-Wallis test was used. Dunn's test was applied for the comparison of the groups.

During the evaluation of the reproduction biology results the number of eggs was normalized with the number of the female having laid the eggs. In the case of the IM F1 specimens, the percentage results were subject to arcsin ($\arcsin(\sqrt{(x/100)})$) transformation for the statistical evaluation of a defined sex ration.

3 Results and discussion

3.1 Tests on adult specimens

During the limit finder tests carried out on adult fish no mortality was experienced in 100 mg/l solution either for 5-FU or for ET treatment.

In Cisp tests, after finishing the 96 hours test LC_{50} value was defined to be 64.5 mg/l for adult fish. IM acute toxicity showed a similar value, LC_{50} value was defined to be 70.8 mg/l for this compound.

3.2 Fish embryo toxicity test (FET) results

During the development of zebrafish embryos 5-FU LC_{50} values were defined in every 24 hours for each tested compound. When testing 5-FU, I could calculate the earliest half-lethal concentration from the mortality data after 72 hours from the fertilization only. The LC_{50} value was 2992 mg/l after 72 hours from fertilization, 2610 mg/l after 96 hours from fertilization and 2222 mg/l after 120 hours from fertilization.

When examining the effects of 5-FU on embryos I detected various deformities. Their occurrence showed a dose-response relation. The half-effective concentration (EC_{50}) calculated based on deformities was 1723 mg/l for 120 hours exposure time. The most characteristic distortion we detected was the bending of the tail in ventral direction (lordosis). This could be detected in concentrations exceeding 1000 mg/l. Similarly, only in case of concentrations over 1000 mg/l was the imperfect closing of the choroid (*fissure choroidea*) in the eyes of the embryos detectable. Such deformations were not detected in either the negative or the solvent-added control groups.

Cisp has proved to be toxic in much lower concentration ranges then 5-FU. Although the LC_{50} value was fairly high after 48 hours exposure time (349.9 mg/l), this value was continuously decreasing as the treatment was going on. After 120 hours of exposure time the LC_{50} value was 81.3 mg/l for larvae. In the case of the 100 mg/l treatment and in higher concentrations all specimens died by the end of the 120 hours exposure time.

As the concentration was increased the ratio of hatched larvae decreased. While the control group hatched after 72 hours as the result of the incubation, this inhibition in hatching was well detectable in concentrations lower than 100 mg/l and it showed a dose-effect relation. No morphological deformation could be detected in animals being still in the egg shells. EC_{50} value calculated for the hatching inhibition was 27.5 mg/l.

During the morphological examination of embryos treated by etoposide various deformities could be detected. These deformities were: bending in

various directions of the rear section of the tail as well as oedema in the yolk and pericardial oedema.

As for the imatinib tests, the LC_{50} value decreased as the exposure time was increased. While LC_{50} was 158.3 mg/l after 48 hours exposure, it was 141.6 mg/l after 72 hours, 118.0 mg/l after 96 hours and 65.9 mg/l after 120 hours. The most characteristic deformity in embryos treated by IM was the bending of the tail in ventral direction. However, other deformities could be detected as well. In case of solutions of 100 mg/l and over for example: reduction in the pigmentation of the tail, distortions in the yolk and occurrence of pigmentation disorders.

3.3 Mixture tests results

The LC_{50} value of Cisplatin/Imatinib mixture calculated during the test of mixtures was 53.1 mg/l in 96 hours, while the LC_{50} value estimated using CA method for Cisplatin/Imatinib mixture was 94.33 mg/l. Estimation using IA method did not predict accurately the combined effect of the mixtures either. The estimated value of the IA method in the $LC_{50}/2$ mixture for the combined effect of the two antineoplastic compounds was 25.8%, while the real mortality value reached 100%.

3.4 Sub-chronic tests

During the sub-chronic tests of 5-FU hatching exceeded 85% in every group after 72 hours from fertilization.

In the entire test period, I detected significant difference in mortality only in the case of the highest concentration (10 mg/l) treatment (92%) (t-test, $p \leq 0.05$). In this way, the lowest measureable toxic effect for mortality took place in the group treated by the 10 mg/l solution. There was no difference in the mortality rate between the solvent-added and negative control groups.

I found verifiable differences when comparing the wet and dry body masses and the standard body lengths during the examination of the three measured body parameters. Based on the dry body mass and the body length parameters the fish treated by 1 mg/l solution (0.695±0.024 mg, 8.644±1.529 mm) were larger on average than the specimens of the control group (0.343±0.08 mg, 6.783±1.4 mm) but this could be verified statistically (Kruskal-Wallis test, Dunns post-hoc test $p \leq 0.05$) only for the body length. As for the wet body mass, I could detect increase in the sizes of fish only in the case of the highest concentration (7.06±0.12 mg) compared to the control group (3.66±0.191 mg)), but this could not be verified statistically (Kruskal-

Wallis test, Dunns post-hoc test $p \leq 0.05$, $p \leq 0.05$). Based on the above, the LOEC value was 1 mg/l treatment for 5-FU.

During the tests carried out in the early life phase of zebrafish treated by imatinib the mortality rate of fish was varying between 36 and 47% between the control group and the 5 lower concentrations. There was no difference in mortality rate between these groups. However, in the treatment carried out using the highest 10 mg/l solution, the mortality rate exceeded 90%, which was already significantly different (t-test, $p \leq 0.05$) from that of the control group. So, as for mortality, the 10 mg/l concentration may be considered to be the LOEC value.

There was no verifiable difference in the examined body parameters between the treated and the control groups.

3.5 Results of the multigenerational tests

As for 5-FU, I detected an outstanding mortality rate of over 50% in generation F1 during the early life phase of the fish (33 dpf) when they were treated by DMSO and the 1 μg/ solution, but this was not statistically verifiable compared to the control group (35.75±8.13 %, Fish exact test, $p \leq 0.05$).

As for F1 specimens, the examination carried out on the 33th day after fertilization on the young fish treated by 5-FU, no statistically verifiable difference could be detected either in the wet or dry body mass or in the body length compared to the control group.

Similarly to the results achieved in the case of F1 specimens, there was no statistically verifiable ($p \leq 0.05$) difference in the wet or dry body mass or in the body length results of the young fish of generation F2 in the control group and the treated group or the solvent-added (DMSO-treated) group.

The tests of generation F1 were finished when the adult fish were two months old already. When we examined the wet body mass there was no detectable difference between the control group (0.195±0.032 mg) and the group treated by DMSO (0.194±0.039 mg). However, in the case of the 10 ng/l treatment (0.163±0.034 mg) the average body mass of the fish was verifiably lower than in the control group (ANOVA, Tukey post hoc test, $p \leq 0.05$). As for the fish treated by the two higher concentration solutions I could detect an opposite effect. In the cases of both the 1 μg/l (0.235±0.038 mg) and the 100 μg/l (0.225±0.044 mg) treatment the average body mass of the fish was significantly higher compared to the control group (ANOVA, Tukey post hoc test, $p \leq 0.05$).

When I examined the average body length of the adult fish, I could not detect any difference between the control group (23.2±1.89 mm) and the group treated by DMSO (22.43±1.6 mm). Only the 1 μg/l (24.14±1.73 mm) treatment showed verifiable difference compared to the group treated by DMSO (ANOVA, Tukey post hoc test, $p \leq 0.05$).

Treatment by imatinib caused also no mortality in generation F0. Mortality detected in the early life phase of generation F1, up to the 33d days from the fertilization showed hardly any difference from the mortality detected in the control group (54.17±4.95 %).

But in the early life phase of generation F2 treated by imatinib a verifiable difference was detected between the mortality of the treated group and that of the control group (51±15.56 %). All treatments done whether by 10 ng/l (86.67±0.94 %), 1 μg/l (78±10.37 %) or 100 μg/l (85±8.96 %) solutions caused significantly higher mortality rates (Fisher exact test, $p \leq 0.05$).

Up to the 33d day of generation F1, there was no detectable difference in the wet and dry body mass measurements between the control group and the groups treated by IM. However, the comparison of the standard body lengths showed that the fish in the groups treated by 1 (6.62±0.93 mm) and 100 μg/l (6.53±1.21 mm) solutions were significantly longer than fish in control (5.80±1.30 mm) group (Kruskal-Wallis test, $p \leq 0.05$).

In the case of generation F2 there was no detectable difference between the wet and dry body masses in the treated groups and in the control groups.

When comparing the body mass of the IM-treated F1 adult fish, we detected verifiable differences between the control group (0.31±0.084 g) and the groups treated by 10 ng/l- (0.245±0.061 g) and 100 μg/l (0.255±0.081 g) solutions (ANOVA, Tukey test, $p \leq 0.05$). As for the body length, the reduced body dimension was statistically verifiable only for the lowest concentration (30.85±1.98 mm). Although the body lengths of the fish treated by 1 μg/l (31.23±2.37 mm) and 100 μg/l-es (31.15±2.62 mm) solutions were smaller, this was verifiably different from the control group (32.23±1.55 mm, ANOVA, Tukey test, $p \leq 0.05$).

4 New scientific results

1. I was the first to define through acute toxicity test the half-lethal concentration of cisplatin and imatinb mesylate for adult zebrafish in case of 96 hours exposure time.

2. I performed acute toxicity tests on zebrafish embryos using the test compounds and I described the sub-lethal effects of 5-fluorouracil (*lordosis, fissure choroidea disorder*). In the case of cisplatin I defined the dose-response relations and I identified the sub-lethal symptoms (hatching inhibition) of the treatment to embryos. Examining the etoposide I identified the following deviations as sub-lethal effects: bending in the rear section of the tail, oedemas in the yolk, pericardial oedemas. In the case of imatinib mesylate I identified a dose-response relation for the mortality and I described the sub-lethal effects of the treatment (reduction in the pigmentation of the tail, various yolk distortions, pigmentation disorders).

3. I was the first to examine the toxic effect of the mixture of cisplatin and imatainib on fish embryos. As my results show, the lethality values of IM and CA estimated using predictive methods presumed a much lower toxicity level than the lethality values, which were detected in the real mixtures. This indicates a synergistic effect.

4. In the case of imatinib, I was the first to examine the sub-chronic toxicity of the substance in the early life phase of zebrafish.

5. I have identified that the exposure of zebrafish to 5-fluorouracil in multiple generations has a verifiable impact on the body length of the adult fish in generation F1.

6. During the multigenerational examination of imatinib I have identified that the mortality rate of the fish was significantly higher in generation F2 in all the 10 ng/l, 1 µg/l and 100 1 µg/l concentrations compared to the control group. Besides that, the body length and body mass of the F1 adult fish treated by 10 ng/l concentration were verifiably smaller than that of the control fish and the body length of the fish treated by 100 µg/l concentration was verifiably smaller than that of the control fish.

7. I have worked out a multigenerational examination method, which is suitable not only for studying the biological impacts on reproduction,

but it also enables the researchers to follow the development of the young fish in generations F1 and F2 and, due to the higher number of fish it can be applied to measure other biological endpoints (for example genotoxicity) as well.

5 Conclusions and suggestions

5.1 Conclusion reached based on the acute tests

Based on the OECD 203 test performed on adult fish, the toxicity level of 5-FU has proved to be low (Category 3 – slightly toxic) as I could not detect any mortality in the tested groups even during the limit test of the 100 mg/l concentration. A similarly low toxicity level (NOEC 1000mg) was identified after 48 hours of exposure in the case of the rainbow trout (*Oncorhynchus mykiss*), a fish, which has different environmental requirements compared to zebrafish. Like in the case of 5-FU, I could not detect any mortality during the limit test of etoposide either. Cisplatin has proved to be the most toxic agent for adult zebrafish from the four antineoplastic compounds.

Therefore, based on the tests I made and the LC_{50} calculated, CisP and IM proved to be moderately toxic (Category 2 – moderately toxic) to the adult fish. The results achieved showed that the toxicity of the four tested compounds is low to the adult fish therefore no fear of acute poisoning in the known environmental concentration ranges is justified. The reason of the low toxicity level is probably the weak absorption ability of the molecules and their short half-life. Besides that, the non-polar molecules might be absorbed by the POC (Particular Organic Carbon) and DOC (Dissolved Organic Carbon) compounds that are present in the waters and in this way the quantity of the absorbable free molecules is reduced further.

Cisplatin proved to be much more toxic to the embryos than 5-FU. The ototoxic effect of Cisplatin to the Zebrafish embryos is well known. The reduction of the LC_{50} value with the increase of the exposure time was obvious during my tests. Beside mortality, I found dose-effect relation between the concentrations and the reduction in the hatching ratio of the larvae. While the LC50 value was 82.3 mg/l in the case of 120 hours exposure, 50% of the living larvae did not hatch in a concentration of 27.5 mg/l. The reason of this reduced hatching ratio might be the ototoxic effect of Cisplatin mentioned already above. With high probability, the hatching inhibition detected in my tests was also due to the reduced moving activity of the fish.

Beside 5-FU, the embryonic toxicity of Etoposide was the lowest among the tested substances. When testing Etoposide, in the case of concentrations over 300 mg/l the crystallization of the compound could be detected on the second day of the test already despite of the presence of the solvent. During my test the LC50 value was over 300 mg/l both for 96 hours and 120 hours exposures. I could state that the embryonic toxicity of Etoposide is also very

low although I could detect the occurrence of various embryonic deformities in the tested concentrations.

Unlike the case of the adult fish, Imatinib proved to be more toxic than Cisplatin during the FET test. In this case too, I could detect the reduction in the LC50 value with the increasing exposure time. Various deformities occurred in the developing embryos in the different concentrations. The toxic effect of Imatinib to the fetal development is well known in mammal models. Based on the data given in the safety documentation of Gleevec®, it caused missing skull bones and disorders in the brain development (anencephaly) in the case of rats in quantities of 100 mg/ttm kg and over (this is a quantity equal to the maximal human dose of 800 mg/day). Unfortunately no comparable results of acute tests on fish are available for Imatinib either.

5.2 Conclusions reached based on the mixture tests

During the tests of Cisplatin and Imatinib mixtures it has become clear that the toxicity of the mixtures was much higher than it could have been expected based on the separate tests of the individual substances. None of the predictive models applied did predict the high mortality rate detected in the tested mixtures. The reasons could be various. These models were elaborated mainly for the estimation of sub-lethal effects, which can be tested in low concentration ranges. In my case however, the mortality was tested in high concentration ranges, which are considered raw endpoints.

It seems based on my results and the data in the literature that, as the tested cytostatics act through different molecular routes, the effects of the two compounds are independent from each other. However, this does not mean that these effects could not add up and could not intensify each other. This could be established from the mortality data of the Zebrafish embryos as well.

5.3 Conclusions reached based on the sub-chronic tests

I performed sub-chronic tests for two substances. The reason to test 5-FU despite of its low toxicity level was that it is the most frequently used antineoplastic medicine in the developed countries presently. The other substance I examined in the sub-chronic test was Imatinib, where I detected the highest mortality rate during the acute embryo tests. When testing these compounds, in both cases I tried to select test concentrations, which enabled me to model the measured and estimated environmental concentrations described in the literature.

During the sub-chronic tests of 5-FU I did not detect deformities in the developing embryos in the tested concentrations. By the end of the treatment I detected significant increase in the mortality in the 10 mg/l concentration only (92±2.8%). There was a statistically verifiable increase in the body length of the fish in the case of the 1 mg/l concentration. As for the treatment in 10 mg/l solution performed during my tests, the fish may have become larger due to reduced population density in the basin resulted by the high mortality rate. This is why the body parameter results of this treatment were not considered during evaluation. However, in the case of the 1 mg/l treatment mortality could not be an explanation to the larger body length of the fish. In the case of the wet and dry body mass such increase was not verifiable. It seems from the results that the long exposure to the anti-metabolic effect of the 1 mg/l 5-FU concentration has interfered with the development of the animals and it showed in the body growth as well.

As to the examination of Imatinib for long term, I detected significant increase in the mortality only in the case of the 10 mg/l treatment. There was no detectable difference in the body parameters of the fish compared to the control group. During the FET test IM has proved to be the most toxic substance, but no impact of the treatment could be detected in the 1 mg/l concentration range after 33 days either.

5.4 Conclusions reached based on the multigenerational tests

The first step of the protocol designed for the multigenerational tests was the pretreatment of generation F0. This was done in the same way for both tests and no mortality was detected in any of the tests. In both tests, in the early phase of generation F1, about half of the fish survived whether they were living in the treated or in the control group. Only in the case 5-FU, the DMSO and 1 µg/l groups showed some deviation from this pattern. For Generation F2 the mortality rate was higher than in Generation F1 in the same life phase in both cases, when the fish were treated by Fluorouracil or Imatinib. In the case of Imatimib this was also statistically verifiable for all of the three concentrations. The most probable explanation for the higher mortality is the reduced vitality occurring due to the treatment because 5-FU as an anti-metabolite can interfere with the S phase of the cell cycle and in this way it can cause disorders in the renewal of the cells. Besides that, the genotoxic impact of 5-FU to Zebrafish models in case of long exposure is known (and this can also reduce the vitality of the animals). However, I have to mention that during the 5-FU tests the mortality rate of generation F2 increased in the control group as well, while it did not change in the group

treated by DMSO. There are several possible explanations for the mortality detected in the case of specimens treated by IM as Tyrosine kinase exerts its inhibitor effect in multiple routes, but only few data about its long term consequences are available. Several tests on patients suffering from both myleoid leukemia and diabetes type 2 showed that the treatment by Imatinib was successful in both diseases. This meant that Imatinib had an impact both on the insulin resistance and on the glucose production of the liver. Based on the above it is possible that the treatment using low concentrations as I applied it may have changed the utilization of glucose in the fish and this may have affected the metabolic processes of the animals and in this way their vitality and growth potential as well.

In the case of the multigenerational tests I could state that the survival rate of the fish in the treatment system applied during their early life phase did not exceed 65% in the control groups either. The reason of the higher mortality rate in my tests (beside the possible injuries caused by the necessary movements) was that the number of the fish involved in the test was at the maximum of the fish keeping capacity of the test setup applied (half-static system, size of the testing vessels). The method used did not allow me to keep more fish successfully in the available system. This was why I used 150 embryos in each repetition at the beginning of the treatment of generation F1 in the case of the IM tests, which were performed in the second step. It is very difficult to define the starting number of fish for such an especially long term test, especially when one wants to breed the fish further. This requires the treatment of minimum 100 embryos in the test setup I used.

Further, during the tests I noticed that the period between day 14 and 16 from the fertilization is most critical during the early life phase of the fish. Most probably this is the period when the yolk sac is fully absorbed at the keeping temperature I used. This means that after this period the fish can get their energy only from the feed they can obtain. As I noticed the survival possibility of fish, which cannot eat Artemia nauplii by this time, is very low should they originate from the treated or from the control group.

As my test results show in the group of 33 dpf old F1 fish, which were treated by 100 µg/l 5-FU solution the average wet body mass was higher, while the average dry body mass was lower compared to the control group.

This suggests that the water content of these fish was higher. This was not the case in generation F2. In this generation the average wet and dry body mass of the fish for all of the treated groups was higher than that of the control groups.

As for groups treated by IM, wet and dry body mass of the fish decreased in generation F1 in the groups treated by either 1 or 100 µg/l solutions. However, the average body length was significantly longer in this case too. This tendency was not detectable already in generation F2. In the case of the two highest concentrations the body length of the fish was larger, but it was not verifiable by statistical methods. It is important to emphasize that the mortality was much higher in all three groups compared to the control group therefore the fish should have grown larger due to the lower population density as it was experienced during the sub-chronic tests of 5-FU in the 10 mg/l group. In contrast, the body mass of the fish in the 10 ng/l group was much lower compared to the control group, while in the case of the two higher concentrations this parameter was almost the same as in the control group or it was slightly higher.

The definition of the body parameters of the adult fish is an important end point in almost all works I found in the literature. During the 5-FU treatment, significant differences could be detected between all treated groups and the control group relating the body mass of the adult fish. But, while the average mass was smaller in the two higher concentrations, this parameter increased in the group treated by 10 ng/l solution. Such a considerable difference was not detectable when I examined the standard body lengths. Comparing these results with the results of the early life phases one can state that the average wet body mass of the fish was larger in the case of the two higher concentrations in all test end points compared to the control specimens. Although this phenomenon was detectable also in the young specimens treated by 10 ng/l solution, in the case of the adult fish a 7 months exposure resulted in the opposite effect. In the case of 5-FU the explanation may be the difference in the concentration ranges (100-fold and 1000-fold). Based on the results received I think it is possible that the molecular and biological processes, which take place in the nanogram ranges are different or have a different intensity level than the ones taking place in the microgram ranges due to the lower quantity of the absorbed 5-FU.

In the case of IM, the treatment clearly caused larger or smaller decreases in the body mass of the adult fish. This decreased body size was verifiable only for 10 ng/l treatments in the case of this compound. Comparing these data with the results measured in the early life phases some difference can be detected. In the case of the shorter exposures the treated groups showed increase rather than decrease in body length. In the case of generation F1 in higher concentrations the body mass of the fish decreased. As for generation F2 however, the body mass of the fish treated by the lowest concentration

was clearly smaller. I think that, like in the case of 5-FU, processes originating from the difference in concentrations might take place in the background although they act through different biological routes.

Considering the results I think that due to the long term exposure both 5-fluorouracil and Imatinib mesylate induced changes in the metabolic processes of the fish. As for 5-FU we tried to find an explanation for this in one of our previous works, in a toxicogenomic examination. We found that mainly the genes related to the apoptotic processes and DNS-repairing mechanisms were over-regulated (bc12, xrcc5, etc.), while many genes related to the protein synthesis were under-regulated. (cth, etc.). Although there were processes, which were over-regulated only in the nanogram concentration ranges (like signal transduction processes, immune processes) and not in the microgram ranges. We saw examples to the opposite phenomenon as well, where the genes were under-regulated in the lower ranges (like trans-membrane processes, ribosome biogenesis) and there were no detectable impacts in the higher concentration ranges. However, we never met a case where some processes would be over-regulated in one range and under-regulated in the other range. IM as a Tyrosine kinase inhibitor however, affects many biologically important routes, although such results are not yet available for me.

5.5 Suggestions:

- The acute embryo toxicity tests described in this study were performed based on a draft of the FET test at that time. The final FET protocol introduced in 2013 also recommends that the treatment of the fish lasts until they reach the age of 96 hours. I think extension of the test period to 120 hours would offer many advantages. As the Zebrafish larvae hatch between 72 and 90 hours depending on the incubation temperature it might happen in the case of a 96 hours exposure that not all specimens can hatch within the time limit even in the control group. This problem could be clearly solved by extending the treatment time by 24 hours and in this way the researcher could get more accurate data about the impact of the tested molecule on the hatching. A further advantage of the extension would be that movement activity test (locomotor response) could be performed on the 120 hours old already swimming fish. The required systems, which are suitable

for quantitative measurements are available in a growing number of laboratories.

- In order to reduce the distorting effect occurring in the results due to the cyclicality in the quantity of the laid eggs, at least 5 breeding cycles must be done in the frame of a toxicology test, which examines also the reproduction ability of the zebrafish.
- During the testing works of the mixtures we found that mortality as an end point is not perfect for studying the effects of the mixtures. A sub-lethal end point must be selected for the zebrafish embryos too, which would represent well the effects of the tested substances. On the other hand, such a sub-lethal effect test would require the modification of the FET test so, that the ratio of the occurred deformities could be examined in multiple steps. A possible solution might be for the FET test the measurement of the fish body lengths. In this way the groups could be compared based on an easily quantifiable parameter of the surviving fish.
- Based on the multigenerational tests we found that long term effects on the aquatic organisms must be considered even in nanogram ranges for both compounds. This is why I would strongly suggest performing at least a full life cycle test on fish model for the cytotoxic substances at a concentration near the estimated environmental concentration (PEC). According to the present regulation of EMA (European Medicines Agency) Phase II. Tier A tests must be performed only when the PEC value is 10 ng/l or higher including also the early life phase of the fish. However, as it was obvious from the sub-chronic tests, these tests do not always provide sufficient information about the long term impacts of compounds with such strong biological effects.

Publications related to the topic of the thesis

Publications in scientific journals:

R. Kovács, K. Bakos, B. Urbányi, J. Kövesi, Gy. Gazsi, A. Csepeli, A. János Appl, D. Bencsik, Zs. Csenki, Á. Horváth (2015): Acute and sub-chronic toxicity of four cytostatic drugs in zebrafish. *Environmental Science and Pollution Research*, 23(15): 14718–14729. (IF 2,828)

R. Kovács, Zs. Csenki, K. Bakos, B. Urbányi, Á. Horváth, V. Garaj-Vrhovac, G. Gajski, M. Gerić, N. Negreira, M. L. de Alda, D. Barceló, E. Heath, T. Kosjek, B. Žegura, M. Novak, I. Zajc, Š. Baebler, A. Rotter, Ž. Ramšak, M. Filipič (2015): Toxicity of low doses of 5-fluorouracil in zebrafish (*Danio rerio*) after chronic exposure to low doses of 5-fluorouracil in a two-generation study. *Water Research*, 77: 201-2012. (IF: 5,528)

Kovács R., Urbányi B., Kovács B., Bencsik D., Staszny Á., Hegyi Á., Csenki Zs. (2009): A zebradánió (*Danio rerio*) mint a toxikológiai vizsgálatok modellállata. *AWETH*, 5(4): 446-447.

Conference abstracts and proceedings:

Kovács R., Bakos K., Reining M., Appl Á., Ferincz Á., Horváth Á., Csenki Zs., Filipic M., Urbányi B. (2015): Különböző anti-neoplasztikus gyógyszermolekulák rövid és hosszú távú hatásának vizsgálata zebradánió (*Danio rerio*) modellen. TOX'2015 Konferencia, 2015 október 14-16., Harkány

Kovács R., Bakos K., Reining M., Appl Á., Gazsi Gy., Garaj-Vrhovac V., Ferincz Á., Horváth Á., Csenki Zs., Filipic M., Urbányi B. (2015): Különböző anti-neoplasztikus gyógyszer molekulák rövid és hosszú távú hatásának vizsgálata zebradánió (*Danio rerio*) modellen. V. Ökotoxikológiai Konferencia, 2015. november 20., Budapest

Kovács R., Csenki Zs., Tarcai Zs., Bencsik D., Gazsi Gy., Bakos K., Kovács B., Gajski G., Gerić M., Garaj-Vrhovac V., Urbányi B., Horváth Á., Filipic M. (2013): Az 5-fluorouracil krónikus toxicitásának vizsgálata hal modellen, két generáción keresztül. XXVII. Halászati Tudományos Tanácskozás, 2013. május 22-23., Szarvas

Kovács R., Tarcai Zs., Gazsi Gy., Bencsik D., Bakos K., Kovács B., Urbányi B., Filipič M., Csenki Zs., Horváth Á. (2012): Citosztatikumok

hatásának vizsgálata zebra dánió (*Danio rerio*) modellen. XXXVI. Halászati Tudományos Tanácskozás, 2012. május 23-24., Szarvas

Kovács R., Csenki Zs., Bakos K., Kovács B., Urbányi B., Horváth Á. (2012): Acute toxicity of cytostatic drugs on zebrafish (*Danio rerio*). 1st CytoThreat workshop on the effects of residues of cytostatics and other pharmaceuticals on non□target organism, 2012. október 16-18., Nápoly, Olaszország

Kovács R., Csenki Zs., Tarcai Zs., Gazsi Gy., Bencsik D., Bakos K., Kovács B., Urbányi B., Filipič M., Horváth Á. (2012): Effects of 5-fluorouracil and cisplatin to the embryonic and early life stage development of zebrafish (*Danio rerio*). Fish and amphibian embryos as alternative models in toxicology and teratology, 2012. október 23-24., Aulnay-sous-Bois/Párizs, Franciország

Publications not related directly to the topic of the thesis

Publication in scientific journals:

Balázs, A., Krifaton, Cs., Orosz, I., Szoboszlay, S., **Kovács, R**., Csenki, Zs., B. Urbanyi, Kriszt, B. (2016): Hormonal activity, cytotoxicity and developmental toxicity of UV filters. *Ecotoxicology and environmental safety*, 131: 45-53. (IF:F 3,130)

Szabó E.R., Plangár I., Tőkés T., Mán I., Polanek R., **Kovács R**., Fekete G., Szabó Z., Csenki Zs., Baska F., Hideghéty K. (2016): l-Alpha Glycerylphosphorylcholine as a Potential Radioprotective Agent in Zebrafish Embryo Model. *Zebrafish*, 13(6): 481-488. (IF 2,175)

Gazsi Gy., Baska F., Baska B., Csenki Zs., Kövesi J., Appl Á. J., Bakos K., Csepeli A., Reining M., **Kovács R.** (2014): A labormodell zebradánió (*Danio rerio*) legfontosabb betegségei: Irodalmi áttekintés és saját eredmények. *Magyar Állatorvosok Lapja*, 136:677–687. (IF:0,185)

Csorbai B., Pereszlényi Á., **Kovács R**., Urbányi B., Horváth L. (2014): The habitat use and selectivity by topmouth gudgeon (*Pseudorasbora parva*). *Acta Zoologica Academiae Scientiarum Hungaricae*, 60(4): 389-400. (IF:0,5)

Staszny Á., Havas E., **Kovács R**., Urbányi B., Paulovits G., Bencsik D., Ferincz Á., Müller T., Specziár A., Bakos K., Csenki Zs. (2013): Impact

of environmental and genetic factors on the scale shape of zebrafish *Danio rerio* (Hamilton 1822): a geometric morphometric study. *Acta Biologica Hungarica*, 64(4): 462–475. (IF:0,504)

Bakos K., **Kovács R**., Staszny Á., Kánainé Sipos D., Urbányi B., Müller F., Csenki Zs., Kovács B. (2013): Developmental toxicity and estrogenic potency of zearalenone in zebrafish (*Danio rerio*). *Aquatic Toxicology*, 136–137: 13-21. (IF: 3,513)

Rácz G., Csenki Zs., **Kovács R.**, Hegyi A., Baska F., Sujbert L., Zsákovics I., Kis R., Gustafson R., Urbányi B., Szende B. (2012): Subacute toxicity assessment of water disinfection byproducts on zebrafish. *Pathology & Oncology Research*, 18: 579-584. (IF:1,555)

Csenki Zs., Zaucker A., Kovács B., Hadzhiev Y., Hegyi Á., Lefler K.K., Müller T., **Kovács R**., Urbányi B., Váradi L., Müller F. (2010): Intraovarian transplantation of stage I-II follicles results in viable zebrafish embryos. *The International Journal of Development Biology,* 54: 585-589. (IF:2,856)